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The Influence of Age, Race, and Gender on Peripheral Blood Mononuclear-Cell Subsets in Healthy Nonsmokers

D. J. TOLLERUD,[1,2] J. W. CLARK,[1] L. MORRIS BROWN,[1] C. Y. NEULAND,[3] L. K. PANKIW-TROST,[3] W. A. BLATTNER,[1,4] and R. N. HOOVER[1]

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To investigate the influence of age, race, and gender on the cellular immune system, we determined T-cell, B-cell, monocyte, natural killer (NK)-cell, and HLA-DR$^+$-cell subsets in 266 nonsmokers from a population-based random sample of healthy adults using monoclonal antibodies and flow cytometry. Blacks had a lower total white blood-cell count than whites ($P \leq 0.0001$), due primarily to a decrease in granulocytes. There was no significant difference in absolute lymphocyte count between blacks and whites. Blacks had a higher proportion of CD19$^+$ cells (Leu 12$^+$ B cells) and a lower proportion of CD3$^+$ cells (OKT3$^+$ T cells) than whites ($P \leq 0.01$). Female sex and increasing age were independently associated with an increased percentage of CD4$^+$ cells (OKT4A$^+$ helper–inducer T-cell subset), resulting in a higher helper/suppressor ratio among women and older individuals ($P \leq 0.05$). Black race and increasing age were independently associated with an increased proportion of HLA-DR$^+$ cells ($P \leq 0.0001$) which was not attributable to B cells or monocytes. No significant age, race, or gender effects were observed for CD14$^+$ cells (Leu M3$^+$ monocytes) or CD16$^+$ cells (Leu 11A$^+$ natural killer cells). These data demonstrate that age, race, and gender are each associated with significant differences in peripheral blood mononuclear-cell subsets. Population-based data such as these provide an important foundation for future design and interpretation of human flow cytometry data.

KEY WORDS: Lymphocyte subsets; normal values; flow cytometry; age, race, gender.

[1]Environmental Epidemiology Branch, National Cancer Institute, Bethesda, Maryland.
[2]Current address: Channing Laboratory, 180 Longwood Avenue, Boston, Massachusetts 02115.
[3]Current address: Braton Biotech, Rockville, Maryland.
[4]Address reprint requests to W.A. Blattner, MD, National Cancer Institute, Executive Plaza North, Room 434, Rockville, Maryland 20892.

INTRODUCTION

Recent technological advances in monoclonal antibody production and flow cytometry have allowed these techniques to be utilized in a wide variety of clinical and epidemiological settings to better define cellular aspects of the immune system (1, 2). However, proper interpretation of immunologic perturbations encountered in the investigational setting requires a detailed knowledge of the range of "normal" or expected values in the study population. Data on expected values for peripheral blood mononuclear-cell (PBMC) subsets is beginning to accumulate (3–9), but most studies to date have relied on small or highly selected populations (e.g., blood donors) with limited clinical, demographic, and socioeconomic characterization.

To investigate the influence of demographic and socioeconomic factors on PBMC subsets in healthy individuals, we studied a large population-based random sample of healthy nonsmoking adults from the Washington, DC, metropolitan area. Data are presented on the effects of age, race, gender, and socioeconomic status on the leukocyte count and differential and on the proportion of T-cell subsets, B cells, monocytes, natural killer cells, and HLA-DR$^+$ cells.

METHODS

Study Population

Random digit dialing was utilized to select a population-based stratified random sample of subjects in the Washington, DC, metropolitan area. Demographic, life-style, and medical information was collected through telephone and self-administered questionnaires. Approximately one-third of

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potential study subjects were excluded on the basis of life-style characteristics (homosexual activity, intravenous drug use) or medical conditions (recent hospitalization, severe allergies, steroid medications, blood product transfusion since 1975, connective tissue disease, or recent pregnancy) which might affect the immunologic parameters under investigation. Racial groups other than whites and blacks were excluded due to the small number of subjects predicted from census data. Included in this report are results for the 266 nonsmoking adult study subjects, aged 20–69 years, selected under this protocol. The participation rates were 79.2% for the telephone interview and 64.2% for the phlebotomy.

Specimen Handling and Cryopreservation

Phlebotomy was performed by a nurse-phlebotomist at a specially equipped mobile van. Blood samples were submitted to a commercial laboratory for routine hematology analysis. Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood by Ficoll–Hypaque density-gradient centrifugation, washed and resuspended in modified RPMI 1640 medium, and cryopreserved as previously described (10). Samples were stored in the vapor phase of a liquid nitrogen freezer until needed for flow cytometry analysis.

Monoclonal Antibodies

The following directly fluorescein-conjugated monoclonal antibodies, purchased from Ortho Diagnostics, Raritan, NJ (ORTHO), or Becton Dickinson Monoclonal Center, Mountain View, CA (BD), were utilized: OKT3 ($CD3^+$ T cells; ORTHO) (11, 12); OKT4 and OKT4A ($CD4^+$ helper–inducer T-cell subset; ORTHO) (11, 13–17); OKT8 ($CD8^+$ suppressor–cytotoxic T-cell subset; ORTHO) (12, 14); anti-Leu 12 ($CD19^+$ B cells; BD) (18); anti-Leu M3 ($CD14^+$ monocytes; BD) (19, 20); anti-Leu 11A ($CD16^+$ natural killer cells; BD) (21); anti HLA-DR (nonpolymorphic HLA-DR antigen; BD) (22, 23); and mouse IgG1 (clone 11-63; BD) and IgG2(a+b) (clones 11-4.1 and MPC-11; BD) as negative control reagents. The surface antigen recognized by anti-HLA-DR is reported to be present on the surface of B cells, monocytes, and activated T cells (22, 23). To help separate the contribution of each cell type to the pool of $HLA\text{-}DR^+$ cells, the relative proportion of activated T cells (ACT-T) was estimated by subtracting from the $HLA\text{-}DR^+$-cell pool the number of B cells and the number of monocytes not eliminated by right-angle scatter gating.

Flow Cytometric Analysis

Prepared samples were analyzed on a fluorescence-activated cell sorter, FACS II (Becton Dickinson, Mountain View, CA) interfaced to a PDP 11/24 DEC computer (Digital Equipment Corporation, Landover, MD). Viable lymphocytes were selected for fluorescence analysis using a combination of forward- and right-angle scatter. The forward-angle light-scatter window was set to exclude electronic noise, debris, and damaged or dying cells, while the right-angle light-scatter window excluded monocytes. Standard window settings were determined for each monoclonal antibody. After gating, only $1.9 \pm 0.1\%$ of cells were Leu $M3^+$, with no significant differences between men and women or between blacks and whites. For analysis of CD 14^+ cells (monocytes), the right-angle light-scatter window was opened to allow for viewing of all mononuclear cells (24). Ten thousand gated events were collected for each monoclonal antibody tested. The percentage of immunofluorescence-positive cells was determined by subtracting the negative control fluorescence from each monoclonal reagent, determined by analysis of cells stained with mouse IgG of the appropriate isotype for the monoclonal reagent tested. A series of quality-control experiments was carried out to evaluate intraanalysis variation. Reproducibility was excellent, with a coefficient of variation ranging from 2 to 5% for the major T-cell subsets to a maximum of 15% for NK cells and B cells.

Statistical Analysis

Comparison of categorical variables were made by chi-square analysis, and Student's t tests were used to compare mean values for continuous variables by selected population characteristics (e.g., age, race, gender). The distribution of mononuclear-cell subset proportions were unimodal, symmetric, and approximately normally distributed, except for $OKT4^+$ cells as described under Results. To correct for minor deviations from a normal distribution, analyses were also performed using log-transformed values for leukocyte count and arcsin-transformed values for mononuclear-cell subset proportions. The resulting significance estimates

Table I. Age, Race, and Gender Distribution of the Study Population (N = 266)

Age group (years)	White		Black		Total
	Men	Women	Men	Women	
20–29	12	16	17	8	53
30–39	23	16	8	14	61
40–49	25	15	9	10	59
50–59	23	16	7	8	54
60–69	14	14	5	6	39
All Ages	97	77	46	46	266

were similar to the analyses presented for the nontransformed values. Linear regression analysis was employed to evaluate the relative contributions of age, race, gender, and other independent variables on white blood cells and mononuclear-cell subset proportions (25).

RESULTS

Population Characteristics

The age, gender, and racial distribution of the study population is shown in Table I. All subjects were nonsmokers without serious health problems. Routine chemistry and hematology studies were performed to confirm normal blood counts, liver function, and renal function.

Leukocytes

Blacks had a lower total white blood cell (WBC) count than whites (P = 0.0001), due to a lower number of neutrophils, monocytes, and eosinophils (Fig. 1). The number of circulating lymphocytes was nearly identical in blacks and whites (P = 0.6). Men had a slightly higher proportion of eosinophils

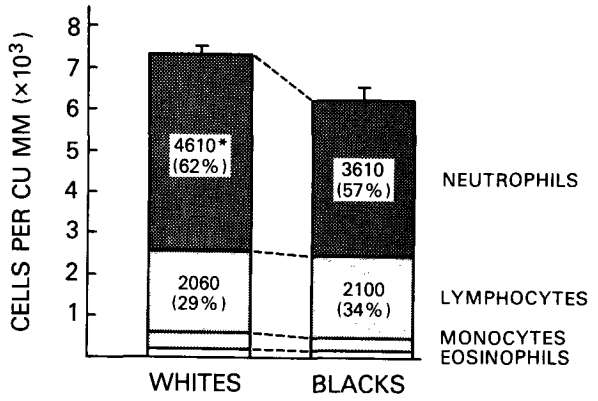

Fig. 1. White blood cell (WBC) count and differential in whites vs blacks. Number of neutrophils and lymphocytes (cells per mm^3) given for blacks and whites and expressed as a percentage of WBC in parentheses. Data for monocytes: whites, 480 (7%); blacks, 380 (6%). For eosinophils: whites, 140 (2%); blacks, 110 (2%). ($^*P \leq < 0.0001$ compared to blacks).

than women (2.1 ± 0.1 vs 1.7 ± 0.1%; P = 0.01), independent of race. The remainder of the leukocyte count was similar in men and women, and there were no significant age-associated differences.

Mononuclear-Cell Subsets

The range and distribution of PBMC subsets in the combined study population are shown in Table II. Blacks had a significantly lower proportion of $CD3^+$ ($OKT3^+$) cells and a higher proportion of $CD19^+$ ($Leu\ 12^+$) cells and $HLA\text{-}DR^+$ cells than whites (Table III). The increase in $HLA\text{-}DR^+$ cells among blacks could not be accounted for by B cells and ungated monocytes alone; the computed estimate of activated T cells (ACT-T) was also significantly increased among blacks. No significant differences between blacks and whites were detected

Table II. Population Distribution of Mononuclear-Cell Subsets

Cell surface antigen	Monoclonal antibody	Cell Proportion[a]			Cell number[b]		
		Mean	SD	Range	Mean	SD	Range
CD3	OKT3	75.1	7.2	48.3–85.3	1582	416	450–2433
CD4	OKT4A	49.3	7.0	29.4–63.3	1036	296	294–1590
CD8	OKT8	28.4	5.4	19.4–38.4	595	174	222–914
CD4:CD8 ratio		1.83	0.54	0.83–3.04	1.83	0.54	0.83–3.04
CD19	Anti-Leu 12	11.0	3.9	4.0–19.3	225	88	58–465
CD14	Anti-Leu M3[c]	18.2	7.2	7.9–50.3	371	127	141–715
CD16	Anti-Leu 11A	6.2	4.7	0.5–24.1	130	106	10–507
HLA-DR	Anti-HLA-DR	14.2	4.9	5.7–29.2	293	121	126–693

[a]Percentage of lymphoid cells.
[b]Cells per cubic millimeter.
[c]Right-angle light-scatter gate removed for determination of Leu $M3^+$ cells.

Table III. Mononuclear-Cell Subsets in Whites vs Blacks[a]

Cell surface antigen	Monoclonal antibody	Whites	Blacks	*P* value[b]
CD3	OKT3	7.6±0.05	73.6±0.8	0.01
CD4	OKT4A	52.2±0.6	51.0±0.7	0.2
CD8	OKT8	27.0±0.5	26.7±0.6	0.7
CD4:CD8 ratio		2.16±0.08	2.04±0.07	0.2
CD19	Anti-Leu 12	10.0±0.3	11.8±0.4	0.0004
CD14	Anti-Leu M3[c]	17.0±0.4	17.5±0.8	0.5
CD16	Anti-Leu 11A	6.7±0.4	6.5±0.4	0.6
HLA-DR	Anti-HLA-DR	14.0±0.4	17.9±0.6	0.0001
	ACT-T	2.1±0.4	4.0±0.6	0.006

[a]Value expressed as mean ± SE percentage of lymphoid cells.
[b]Student's two-tailed *t* test.
[c]Right-angle light-scatter gate removed for determination of Leu M3$^{\pm}$ cells.

for the major T-cell subsets, CD14$^+$ (Leu M3$^+$) cells or CD16$^+$ (Leu 11A$^+$) cells.

Women had a significantly higher proportion of CD4$^+$ (OKT4A$^+$) cells, a lower proportion of CD8$^+$ (OKT8$^+$) cells, and a higher ratio of CD4$^+$ to CD8$^+$ cells (helper–suppressor ratio) than men (Table IV). A similar pattern was observed in whites and blacks analyzed separately. Gender-related differences were not apparent for the other monoclonal antibodies tested.

The influence of age on PBMC subset levels was analyzed using a linear regression model controlling for race and gender effects (Table V). Increasing age was associated with an increased proportion of CD4$^+$ cells and a decreased proportion of CD8$^+$ cells, resulting in an age-related increase in the helper:suppressor ratio. Increasing age was also associated with an increase in percentage of HLA-DR$^+$ cells which remained significant after subtracting the contribution of CD19$^+$ cells (B cells) and gated CD14$^+$ cells (monocytes) (Fig. 2).

A more detailed analysis of age-related changes revealed a significant decrease in the proportion of CD4$^+$ cells among older black women compared to white women (Fig. 3). The CD4$^+$-cell level fell from 52.8 ± 1.1% for black women under age 50 to 48.0 ± 2.0% for those age 50 or older ($P < 0.05$). Among white women, CD4$^+$-cell levels reached a plateau but did not significantly decrease after age 50. No such discontinuity was noted for men (Fig. 4).

Deficiency of the T4 Epitope Among Blacks

The cell surface antigen which identifies the helper–inducer T-cell subset is composed of several antigenic determinants or epitopes. OKT4 and OKT4A bind to different epitopes, and some black subjects with a normal proportion of OKT4A$^+$ cells have no detectable OKT4$^+$ cells. This has been interpreted as deficient expression of the T4 epitope (26). To investigate black:white differences in expression of the T4 epitope, sequential analyses using OKT4 and OKT4A were performed on all samples. For most subjects, the fluorescence intensity distribution for OKT4$^+$ cells was similar to that for OKT4A$^+$ cells, indicating full expression of the

Table IV. Mononuclear-Cell Subsets in Males vs. Females[a]

Cell Surface antigen	Monoclonal antibody	Men	Women	*P* value[b]
CD3	OKT3	74.8±0.06	75.8±0.7	0.3
CD4	OKT4A	50.7±0.6	53.1±0.7	0.01
CD8	OKT8	27.8±0.6	26.0±0.6	0.03
CD4:CD8 ratio		2.01±0.07	2.25±0.09	0.04
CD19	Anti-Leu 12	10.8±0.4	10.5±0.3	0.6
CD14	Anti-Leu M3[c]	17.7±0.6	16.6±0.5	0.2
CD16	Anti-Leu 11A	6.8±0.3	6.4±0.3	0.5
HLA-DR	Anti-HLA-DR	15.7±0.5	15.0±0.5	0.3
	ACT-T	3.0±0.5	2.5±0.4	0.5

[a]Values expressed as mean ± SE percentage of lymphoid cells.
[b]Student's two-tailed *t* test.
[c]Right-angle light-scatter gate removed for determination of Leu M3$^{\pm}$ cells.

Table V. Multiple Linear Regression Analysis of the Effects of Age, Race, and Gender on Mononuclear-Cell Subsets[a]

Cell surface antigen	Intercept	Coefficient			Model R^2
		Age (*P*)	Race (*P*)	Gender (*P*)	
CD3	77.7	−0.009 (0.78)	−2.73 (0.0057)	1.14 (0.22)	0.03
CD4	45.6	0.092 (0.0095)	−1.00 (0.32)	2.43 (0.0103)	0.06
CD8	33.13	−0.063 (0.0441)	−0.53 (0.55)	−1.89 (0.0253)	0.04
CD4:CD8	1.30	0.013 (0.0022)	−0.08 (0.50)	0.25 (0.0279)	0.06
CD19	10.21	−0.029 (0.11)	1.66 (0.0013)	−0.37 (0.44)	0.06
CD14	18.34	−0.009 (0.77)	0.77 (0.36)	−1.25 (0.12)	0.01
CD16	6.61	0.167 (0.37)	−0.10 (0.85)	−0.39 (0.43)	0.01
HLA-DR	5.99	0.110 (0.0001)	4.50 (0.001)	−0.93 (0.11)	0.21

[a]Linear regression model in the form $Y = A + B_1X_1 + B_2X_2 + B_3X_3$, where Y represents the cell subset proportion (%), A is the linear intercept, X_1 represents age in years, X_2 represents race (1 = white, 2 = black), and X_3 represents gender (1 = male, 2 = female), and B_1, B_2, and B_3 are the respective coefficients. A positive coefficient indicates an increase in subset proportion for increasing values of X (e.g., percentage $CD4^+$ cells is higher in females than in males), while a negative coefficient indicates a decrease in subset proportion for increasing values of X (e.g., percentage $CD8^+$ cells decreases with increasing subject age). The P value for each coefficient is given in parenthesis.

T4 epitope. However, two other fluorescence intensity patterns were detected: partial expression, manifested by a normal number of $OKT4^+$ cells with a 50% reduction in mean fluorescence intensity (MFI); and absent expression, with no $OKT4^+$ cells but the normal complement of $OKT4A^+$ (Fig. 5). No deficiencies in T4 epitope expression were detected in white subjects enrolled in this study. However, a trimodal population distribution of OKT4 MFI values was noted for black subjects, with 4.3% absent expression, 26.1% partial expression, and 69.6% full expression. T4 epitope expres-

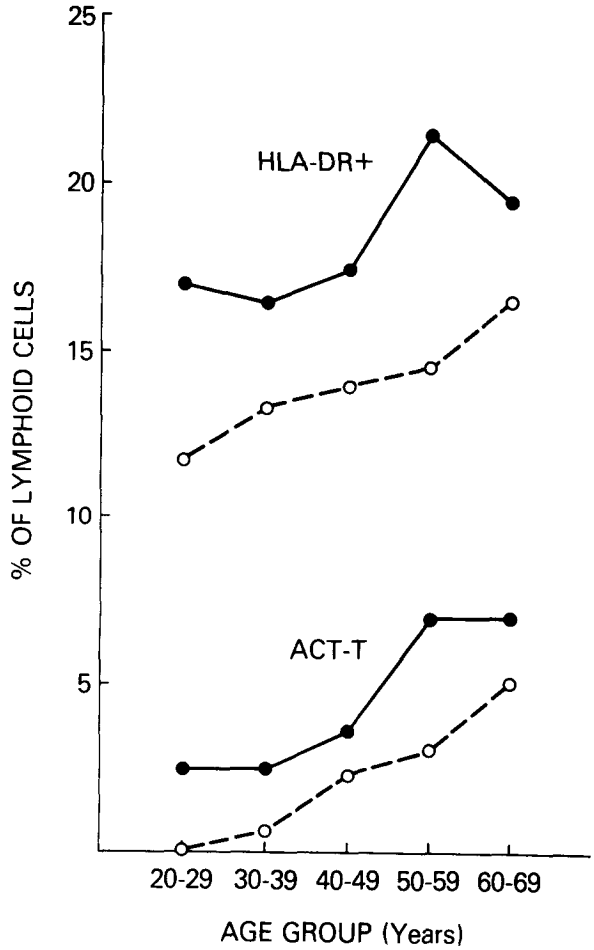

Fig. 2. Influence of race and age on $HLA\text{-}DR^+$ cells and activated T cells (ACT-T). Filled circles, black subjects; open circles, white subjects. $P < 0.0001$ for blacks vs whites.

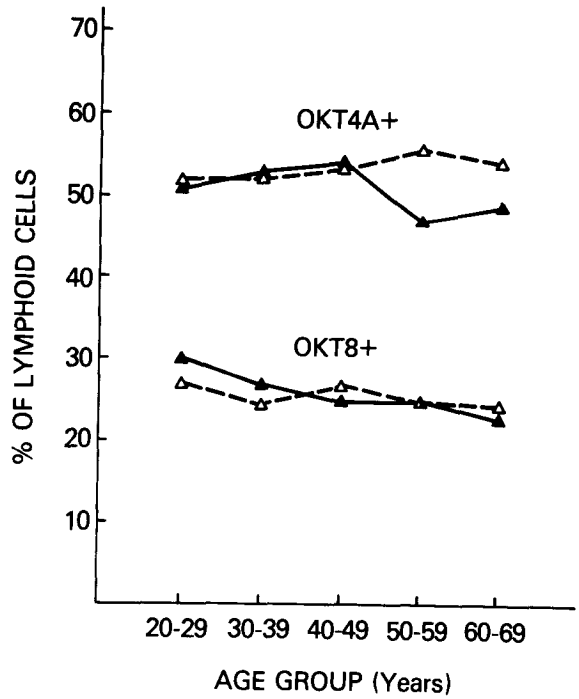

Fig. 3. Influence of race and age on T-cell subsets among women. Filled triangles, black women; open triangles, white women.

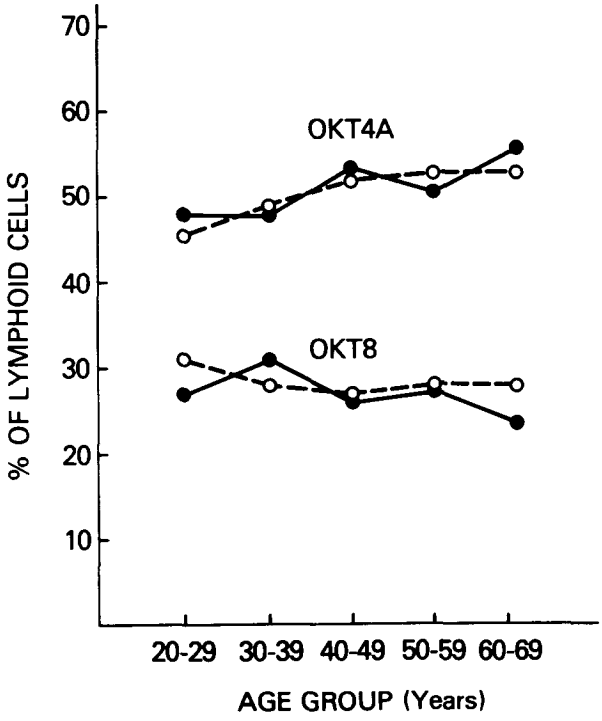

Fig. 4. Influence of race and age on T-cell subsets among men. Filled circles, black men; open circles, white men.

sion was uniform for each subject; no individuals exhibited two populations of $OKT4^+$ cells with different fluorescence intensities.

DISCUSSION

Previous reports of PBMC subsets in "normal" subjects have been dominated by analyses of small or highly selected populations, with little attention to the potential influence of race, socioeconomic factors, or common environmental exposures. The use of tobacco products has seldom been characterized, although cigarette smoking has been clearly associated with alterations in T-cell subsets (27–29). Adequate knowledge of expected values and the effects of fundamental population characteristics is critical for accurate interpretation of PBMC subset data. In the current analysis, the inherent selection bias in studies of laboratory "volunteers" or blood donor populations was avoided by selecting a stratified random sample of the general population in a large metropolitan area. The health status of selected subjects was assessed using responses from detailed telephone and self-administered questionnaires, and individuals with medical conditions or environmental exposures known or suspected to

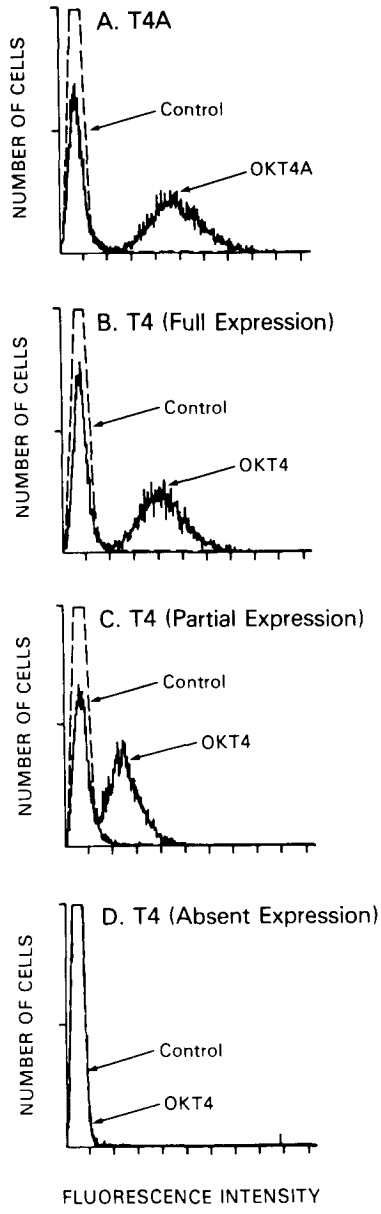

Fig. 5. Representative fluorescence intensity histograms for cells labeled with OKT4 or OKT4A among black subjects. (A) Typical histogram for OKT4A; (B) full expression of the T4 epitope (69.6% of blacks); (C) partial expression of the T4 epitope (26.1% of blacks), with a normal number of $OKT4^+$ cells but mean fluorescence intensity reduced by 50%; (D) absent expression of the T4 epitope (4.3% of blacks), with no detectable $OKT4^+$ cells.

alter PBMC subsets were carefully excluded. Extensive information on current medication use was also collected. Relatively few individuals took prescription medications in this healthy population, and only minor variations in medication use were noted between race/gender strata (Tollerud *et al.*, unpublished observations). For example, antihy-

pertensives were taken by only 8.3% of subjects, including 8.4% of whites and 8.0% of blacks. There were no systematic differences in medication use between men and women or between blacks and whites which might account for the observed differences in mononuclear-cell subsets. The stratification protocol was designed to ensure sufficient numbers of subjects in each age/gender/race category for adequate statistical analysis. To avoid the confounding effects of cigarette smoking, only nonsmokers were included.

As previously reported, blacks had a significantly lower WBC count than whites, due primarily to a decreased number of granulocytes (30). However, the absolute number of lymphocytes per volume of blood was equal in whites and blacks, allowing lymphocyte subset levels in blacks and whites to be compared directly in immunological investigations without concern for race-related differences in total lymphocyte count.

T-cell subset levels varied significantly with age, gender, and race. Women had a higher proportion of $CD4^+$ cells, a lower proportion of $CD8^+$ cells, and a higher helper:suppressor ratio than did men. Increasing age was also associated with an increase in the proportion of $CD4^+$ cells, a decrease in the proportion of $CD8^+$ cells, and an increase in the helper:suppressor ratio. These findings are consistent with results of four recent reports (6–9). However, the racial distribution of those populations was not reported, and only one study mentioned subject's smoking status. Blacks in the present study had a significantly lower proportion of $CD3^+$ cells and a higher proportion of $CD19^+$ cells (B cells) and a than whites. A surprising finding was a significant reduction in the proportion of $CD4^+$ cells among black women over age 50, a phenomenon not observed among white women in the study. The reason for this apparent decline in the $CD4^+$ subset among older black women is not apparent. Such a decline might be due to postmenopausal or other factors not shared with their white counterparts or may be due to chance. For clinical laboratory directors wishing to establish reference ranges, it is reassuring that the age effect for the major T-cell subsets is relatively small. An average 10% increase in $CD4^+$ cells and a 10% decrease in $CD8^+$ cells would be anticipated over the entire five decades from 20 to 70 years. In contrast, the proportion of $HLA\text{-}DR^+$ cells would be expected to double.

The helper:suppressor ratio has been widely studied in patients with the acquired immunodeficiency syndrome (AIDS) (31, 32). A low ratio, particularly when associated with an absolute decrease in the number of $CD4^+$ cells, has been highly correlated with the clinical diagnosis of AIDS. A recent study of helper:suppressor ratios in a large blood donor population suggested that a ratio below 0.85 should raise the suspicion of AIDS (7). The mean helper:suppressor ratio in the present study was 2.1 (95% confidence interval, 1.1–3.8). Two individuals (0.7%) had ratios below 0.85, compared to 1.9% in the blood bank study.

Our data confirm the previously reported heterogeneity in expression of the T4 epitope among black subjects (26). Although no deficiencies in T4 epitope expression were detected among whites in this study, we have previously demonstrated partial T4 expression in one Caucasian (33). This problem is obviated with the use of OKT4A or anti-Leu 2 monoclonal antibodies which appear to bind to an epitope which is uniformly expressed among blacks as well as whites. Among our subjects whose cells expressed the T4 epitope, the age and gender associations noted for OKT4A were similarly observed for OKT4.

Published data on other mononuclear-cell subsets are scarce. This study suggests that the proportions of cells labeled with anti-Leu M3 ($CD14^+$) and anti-Leu 11A ($CD16^+$) are not significantly influenced by age, gender, or race. However, the proportion of $HLA\text{-}DR^+$ cells was significantly higher in blacks than in whites and increased with age in both groups. When $CD19^+$ cells (B cells) and the small fraction of $CD14^+$ cells (monocytes) not excluded by light-scatter gating were subtracting from the $HLA\text{-}DR^+$ cell fraction, significant associations with race and age persisted. The resulting derived variable (ACT-T) provided a crude estimate of the proportion of activated T cells among PBMC. While the calculated ACT-T value was not a quantitatively accurate measure of activated T cells, these data suggest that the proportion of activated T cells increases with age.

The importance of considering demographic characteristics when assessing mononuclear-cell subsets is illustrated by a study of T-cell subsets in patients with idiopathic thrombocytopenic purpura (ITP) reported by Mylvaganam *et al.* (34). They noted T-cell subset differences between men and women in their control subjects which were similar to the results of the present study. Using this information, they analyzed men and women with ITP separately and found significantly gender-

associated differences in T-cell subset imbalances within the patient population. Failure to recognize such male:female differences might have obscured a key finding from their investigation. Even greater difficulties may arise in analyses of cell subsets which show significant variation with more than one demographic characteristic. For example, anti-HLA-DR has been utilized in studies of renal transplant recipients (35). HLA-DR$^+$ cells comprised approximately 20% of lymphoid cells in healthy black subjects over age 50 in the present study. If this group were compared to a laboratory "reference range" for HLA-DR based on analyses of blood samples from young white individuals (12% HLA-DR$^+$ cells), the resulting "statistically significant" difference could be erroneously interpreted as abnormal.

To our knowledge, this is the first report of monoclonal antibody-defined peripheral blood mononuclear-cell subsets in a population-based random sample of healthy nonsmokers. The specific values presented here may not be directly applicable to other populations or to analyses performed by other laboratories, but the associations with age, race, and gender are unlikely to be center specific. Recent reports indicate that T-cell subsets are similar in geographically distinct populations and that flow cytometry data are quite reproducible in different laboratories using similar techniques (36). These analyses provide baseline data for further studies of mononuclear-cell subsets and highlight the need to recognize and control for demographic differences between study populations.

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